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Abstract:	<p>The role of the macrophage in influenza virus infection is complex. Macrophages are critical for resolution of influenza virus infections but implicated in morbidity and mortality in severe infections. They can be infected with influenza virus and consequently macrophage infection is likely to have an impact on the host immune response. Macrophages display a range of functional phenotypes from the prototypical pro-inflammatory classically activated cell to alternatively activated anti-inflammatory macrophages involved in immune regulation and wound healing. We were interested in how macrophages of different phenotype respond to influenza virus infection and therefore have studied the infection of bone marrow derived macrophages (BMDMs) of classical and alternative phenotype in vitro. Our results show that alternatively activated macrophages are more readily infected and killed by the virus than classically activated. Classically activated BMDMs express the proinflammatory markers inducible nitric oxide synthase (iNOS) and TNFα and TNFα expression was further up-regulated following infection. Alternatively activated macrophages express Arginase-1 and CD206, however, following infection, expression of these markers is down regulated while expression of iNOS and TNFα is upregulated. Thus, infection can override the anti-inflammatory state of alternatively activated macrophages. Importantly, however, this results in lower levels of pro-inflammatory markers than those produced by classically activated cells. Our results show that macrophage phenotype affects the inflammatory macrophage response following infection and indicate that modulating the macrophage phenotype may provide a route to develop novel strategies to prevent and treat influenza virus infection.</p>

**Susceptibility of bone marrow derived macrophages to influenza
virus infection is dependent on macrophage phenotype**

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Summary

The role of the macrophage in influenza virus infection is complex. Macrophages are critical for resolution of influenza virus infections but implicated in morbidity and mortality in severe infections. They can be infected with influenza virus and consequently macrophage infection is likely to have an impact on the host immune response. Macrophages display a range of functional phenotypes from the prototypical pro-inflammatory classically activated cell to alternatively activated anti-inflammatory macrophages involved in immune regulation and wound healing. We were interested in how macrophages of different phenotype respond to influenza virus infection and therefore have studied the infection of bone marrow derived macrophages (BMDMs) of classical and alternative phenotype *in vitro*. Our results show that alternatively activated macrophages are more readily infected and killed by the virus than classically activated. Classically activated BMDMs express the proinflammatory markers inducible nitric oxide synthase (iNOS) and TNF α and TNF α expression was further up-regulated following infection. Alternatively activated macrophages express Arginase-1 and CD206, however, following infection, expression of these markers is down regulated while expression of iNOS and TNF α is upregulated. Thus, infection can override the anti-inflammatory state of alternatively activated macrophages. Importantly, however, this results in lower levels of pro-inflammatory markers than those produced by classically activated cells. Our results show that macrophage phenotype affects the inflammatory macrophage response following infection and indicate that modulating the macrophage phenotype may provide a route to develop novel strategies to prevent and treat influenza virus infection.

Introduction

Influenza A viruses impose a considerable burden on human health. Seasonal influenza virus infections range from mild to life-threatening with the outcome dependent on both strain of virus and host response. They are associated with significant morbidity and mortality, particularly in the elderly and the very young. The emergence of highly pathogenic avian strains that can infect humans, albeit with a limited ability to spread human to human, presents an additional threat (Webster & Govorkova, 2014). A key feature of these strains is an overreactive immune response that fails to control the infection, resulting in excessive production of cytokines and chemokines, influx of immune cells and severe immunopathology which contributes to high mortality (Cheung *et al.*, 2002; Kobasa *et al.*, 2007; Korteweg & Gu, 2008).

Influenza virus primarily infects epithelial cells in the respiratory track but also has a well-recognized ability to infect macrophages (Rodgers & Mims, 1982; Tumpey *et al.*, 2005). Macrophages play a central role in initiating and controlling the immune response to infections and infection of these innate immune cells is highly likely to have consequences for the outcome of infection. It is clear, however, that the ability to infect and replicate in macrophages is dependent on both virus strain and origin of the macrophage and that the results of infection are highly variable (Nicol & Dutia, 2014; Short *et al.*, 2012). Infection of monocyte derived macrophages *in vitro* with highly pathogenic viruses leads to production of higher levels of inflammatory cytokines and chemokines than are produced in response to infection with low pathogenicity seasonal viruses (Cheung *et al.*, 2002; Zhou *et al.*, 2006) suggesting that infection of macrophages may be, at least in part, responsible for the increased pathogenicity of these viruses. *In vivo* studies, however, have shown that a virus strain that readily infects macrophages *in vitro* is less pathogenic in mice than a related virus that fails to infect macrophages (Tate *et al.*, 2010). In this instance, depletion of macrophages increases the virulence suggesting that macrophage infection can attenuate pathogenesis.

Macrophages are not, however, a single homogeneous population. They are highly pleiomorphic cells with a range of phenotypes and functions (Gordon & Taylor, 2005). At the extremes of the spectrum of macrophage phenotypes are the “classically activated” or M1 macrophage, generally considered to be pro-inflammatory, producing TNF α , IL-6, IL-1 β and inducible nitric oxide synthase (iNOS), while the “alternatively activated” or M2 macrophage has up-regulated expression of the macrophage mannose receptor CD206 and MHC class II, produces high levels of Arginase-1 (Arg-1) and endocytic function and is considered to be associated with wound healing and repair. IFN γ and TNF α drive classical macrophage activation while alternative macrophage activation is driven by the Th2 cytokines IL-4 and IL-13. Between these two extremes lies a range of subtly different phenotypes which orchestrate and regulate the immune response (Gordon, 2003).

We hypothesized that susceptibility and subsequent response of macrophages to influenza virus infection may depend on their phenotype, and that it would be possible to alter the extent of infection and thus influenza-associated pathology by manipulating macrophage phenotype. Here we show that alternatively activated BMDMs are more susceptible to infection with the A/WSN/33 strain of influenza virus than classically activated BMDMs and are more readily killed by infection. Infection of alternatively activated BMDMs overrides their anti-inflammatory state, inducing a pro-inflammatory macrophage phenotype. However, infection of alternatively activated BMDMs results in production of lower levels of pro-inflammatory markers including iNOS and TNF α than infection of classically activated BMDMs. Overall, our study supports the hypothesis that alternatively activated macrophages have a protective role in highly pathogenic virus infection.

Results

129Sv/Ev bone marrow derived macrophages can be infected with influenza virus strain A/WSN/33

Previous studies have reported that influenza viruses can infect macrophages with varying efficiency in a virus strain dependent manner (Reading *et al.*, 2000; Rodgers & Mims, 1981; Tate *et al.*, 2010). We wished to investigate the ability of A/WSN/33 to infect macrophages from mice on the 129Sv/Ev background, therefore we derived macrophages from femurs of 6-8 week old female 129Sv/Ev mice by culture for 7 days in medium containing M-CSF. FACS analysis showed that >95% of the cells expressed the macrophage markers CD11b and F4/80 (data not shown) and therefore were of macrophage phenotype (Misharin *et al.*, 2013). The bone marrow derived macrophages (BMDMs) were infected with varying multiplicities of infection (MOI) using viral titres determined on MDCK cells, incubated for various lengths of time and stained for viral antigen using a polyclonal antibody directed against purified H1N1 virus. No antigen positive cells were detected at 1h post-infection (Fig1b) but positive cells were detected from 6h post-infection (fig 1c,d) indicating that viral protein synthesis was required for antibody staining. An MOI of 10 resulted in infection of around 60% of cells but increasing the amount of input virus did not increase the percentage of cells infected further. Poisson distribution predicts that, if BMDMs were as infected with the same efficiency as MDCKs, an MOI of 5 should result in infection rate of > 99% of cells. Thus, although BMDM can be infected with A/WSN/33, they are less readily infected than MDCK cells.

Effect of macrophage phenotype on infection

In order to produce polarized macrophage populations, we treated macrophages derived from wild type 129Sv/Ev mice and mice on the same genetic background lacking the IFN γ receptor (IFN γ R^{-/-}) with IFN γ or IL-4 for 16 hours and measured levels of iNOS and Arg-1 by qRT-PCR and biochemical assay. Figure 2 shows that treatment of both 129Sv/Ev and IFN γ R^{-/-} BMDMs with IL-4 (Fig 2a,b) for 16 hours led to induction of Arg-1 mRNA and arginase activity, indicating

that the macrophages had differentiated to an alternatively activated –like phenotype. Treatment of 129Sv/Ev BMDMs with IFN γ resulted in production of iNOS mRNA and iNOS activity (Fig 2c,d) confirming that these BMDMs had differentiated to a classical phenotype. IFN γ R^{-/-} BMDM cannot respond to IFN γ and therefore were not able to produce a classical macrophage response, ie they did not upregulate iNOS upon IFN γ treatment (Fig 2c,d). We next infected the polarized macrophages with A/WSN/33 and stained with antibody to H1N1 virus (Fig 3). Treatment of 129Sv/Ev and IFN γ R^{-/-} BMDMs with IFN γ or IL-4 and infection with virus did not affect the cell density (Fig 3a-c, f-h). However, it was clear from 6hr post-infection that IL-4 treated macrophages were more readily infected than IFN γ treated macrophages (Fig 3d & i). Similar results were observed at 48h post-infection (Fig 3e & j) and intervening time points. Thus, alternatively activated macrophages from both strains of mice are more readily infected than classically activated.

Consistent with the higher levels of viral antigen present in alternatively activated macrophages, qRT-PCR showed significantly higher amounts of M1 mRNA synthesized in these cells indicating higher levels of viral infection ($p < 0.001$) (Fig 4).

Survival of influenza virus infected macrophages

The survival of influenza virus infected BMDMs was assessed by use of the CellTiter-Blue viability assay. Figures 5 a & b show that by 20h post-infection, infection of alternatively activated macrophages resulted in a significantly lower rate of viability than seen in infected classically activated macrophages. Interestingly, 129Sv/Ev IFN γ treated macrophages show a trend towards higher viability than untreated BMDMs from the same mice (Fig 5a). This difference is not apparent for the IFN γ R^{-/-} BMDMS which cannot respond to IFN γ suggesting that classical activation confers protection against virus induced cell death. These data suggest that IL-4 activation renders BMDM more permissive for A/WSN/33 and that once infected, these cells are more readily killed by the virus than classically activated cells. In order

to determine whether higher levels of productive virus infection occurred in alternatively activated macrophages, we measured the infectious virus present in the cell supernatants (Fig 5c). At 48h post-infection, the amount of virus in supernatants was higher than at 1h post-infection indicating that there was a low level replication in the BMDMs. However, the amount of virus recovered was less than the amount of input virus and we found no evidence that alternative activation resulted in production of higher levels of infectious virus by the BMDMs.

Cytokine response to influenza virus infection

In order to assess the effect of influenza virus infection on macrophage phenotype, we analysed the expression of phenotypic markers in infected/polarized BMDMs. iNOS, TNF α and IL-12p40 were chosen as markers of classically activated macrophages. iNOS is associated with inflammatory macrophage response and the secreted cytokines TNF α and IL-12p40 contribute to macrophage driven inflammatory response. In addition to Arg-1, which is highly expressed in alternatively activated macrophages (Fig 2), we measured expression of the mannose receptor, CD206 which is upregulated on alternatively activated macrophages (Gordon, 2003). BMDMs were treated with IFN γ or IL-4 and infected with A/WSN/33 at an MOI of 10. Cells were harvested at 48 hours post-infection and the expression of markers was measured by qRT-PCR. Treatment of 129Sv/Ev macrophages with IFN γ upregulated iNOS by $>10^4$ fold and no further upregulation was induced by infection with virus (Fig 6a). Similar results were found for IL-12p40 (data not shown). In contrast, TNF α expression in these macrophages, although elevated by IFN γ alone, was significantly higher in cells which had been infected with virus ($p<0.05$, Fig 6e). Thus virus infection can drive expression of this pro-inflammatory cytokine. Arg-1 and CD206 expression in the IFN γ treated 129Sv/Ev macrophages was low and virus infection did not alter this (Fig 6c,g). As expected, IFN γ treatment of macrophages grown from IFN γ R $^{-/-}$ mice did not result in significant changes in expression of the classical markers iNOS and TNF α (Fig 6a,e). Infection of IFN γ R $^{-/-}$ macrophages with influenza virus upregulated iNOS expression (Fig 6a) indicating the virus

alone could switch on expression of this inflammatory marker. TNF α expression was clearly completely dependent on the IFN γ activity as there was no change in expression following virus infection of the IFN γ R^{-/-} macrophages (Fig 6e). Similarly, there was no significant change in IL-12p40 expression (data not shown). Thus, infection of classically activated macrophages leads to increased expression of TNF α enhancing the pro-inflammatory state of these macrophages. In the absence of IFN γ responsiveness infection led to enhanced expression of iNOS but did not induce synthesis of secreted pro-inflammatory cytokines (TNF α and IL-12) indicating that these macrophages did not enter a pro-inflammatory state.

Alternative activation of BMDMs from both wild type 129Sv/Ev and IFN γ R^{-/-} mice resulted in upregulation of Arg-1 and CD206 compared to untreated BMDMs (Fig 6d,h). Classical markers were low or undetectable in these cells (Fig 6b,f). However, infection of alternatively activated 129Sv/Ev BMDMs induced expression of iNOS and TNF α indicating that virus infection could override the alternative, anti-inflammatory state of these macrophages, produce a pro-inflammatory state within the cells (iNOS) and led to secretion of pro-inflammatory cytokines (Fig 6b,f). Similarly, iNOS was significantly upregulated following infection of alternatively activated IFN γ R^{-/-} BMDMs (Fig 6b; $p < 0.001$) but levels were lower than those produced in alternatively activated 129Sv/Ev BMDMs. Again, expression of TNF α was completely dependent on responsiveness to IFN γ (Fig 6f). Infection of alternatively activated wild type 129Sv/Ev and IFN γ R^{-/-} BMDMs led to decreased expression of Arg-1 and CD206 (Fig 6 d,h). Thus, infection of alternatively activated macrophages led to down regulation of alternative markers and induction of the pro-inflammatory mediators iNOS and TNF α . However, the levels of these cytokines produced by alternatively activated macrophages are less than those produced by classically activated macrophages. Whilst infection can clearly override the anti-inflammatory state, infection of alternatively activated macrophages leads to lower levels of pro-inflammatory cytokine production than those observed following infection of classically activated macrophages.

Discussion

Our results show that alternatively activated macrophages are more susceptible to infection with A/WSN/33 than classically activated cells. A higher percentage of cells express viral antigens and higher levels of M1 mRNA are produced in alternatively activated cells than in classically activated cells. Similarly, Hoeve *et al* (Hoeve *et al.*, 2012) showed that following infection with the H3N2 virus Udorn, a significantly higher number of human monocyte derived macrophages with anti-inflammatory characteristics contained viral antigen than those with proinflammatory phenotype. The effect of IL-4 on uptake of antigens by macrophages is dependent on both antigen and pathway. Treatment of macrophages with IL-4 leads to increased uptake of soluble antigen as well as increased mannose receptor dependent uptake of antigen (Montaner *et al.*, 1999; Raveh *et al.*, 1998). However, alternative activation of macrophages with IL-4 has been shown to impair phagocytosis of bacteria and microbial particles (Varin *et al.*, 2010). The higher levels of M1 mRNA in IL-4 treated cells argue that the presence of virus antigen in cells is not simply due to increased phagocytosis of viral antigens but rather is due to increased infection of these macrophages. Influenza viruses usually enter cells by endocytosis following initial binding of the HA to sialic acids on the cell surface (Matlin *et al.*, 1981; Skehel & Wiley, 2000). However, there is evidence that influenza A virus can use other cell surface molecules including the macrophage mannose receptor CD206, macrophage galectin type lectins DC-SIGN and L-SIGN to bind to and enter macrophages (Londrigan *et al.*; Reading *et al.*, 2000; Upham *et al.*, 2010). Alternatively activated macrophages express a different range of cell surface proteins to those found on classically activated macrophages. For example, CD206, is more highly expressed on the surface of alternatively activated macrophages than on classically activated and is indeed considered a marker for alternative activation (Gordon, 2003; Stein *et al.*, 1992). At this point, further work is required to understand the mechanisms by which alternatively activated macrophages are more readily infected but this may have important implications for influenza virus pathogenesis.

Interestingly, alternatively activated macrophages are more readily killed by infection with A/WSN/33 than classically activated macrophages. Influenza virus infection leads to cell death hence it is likely that the difference reflects the level of infection. qRT-PCR data show that at 48h post-infection there is up to 100 fold more M1 mRNA in alternatively activated cultures than in classically activated. This, together with the fact that a higher percentage of alternatively activated macrophages is infected is consistent with the higher level of cell death found in alternatively activated cultures. It is notable, however, that the apparently more permissive state of alternatively activated macrophages did not lead to production of higher levels of infectious virus than are found in classically activated cells. Although there is evidence for productive influenza virus infections in human macrophages (Hoeve *et al.*, 2012; Perrone *et al.*, 2008; van Riel *et al.*, 2011; Yu *et al.*, 2011), a number of publications have reported that influenza virus infection is abortive in murine macrophages (Rodgers & Mims, 1981; Tate *et al.*, 2011; Tate *et al.*, 2010). Our data show that whilst there is some replication of A/WSN/33 in murine BMDMs the ability to produce infectious virus is not related to the activation state of the macrophage. The ease with which alternatively activated macrophages become infected has important implications, i.e. manipulation of phenotype *in vivo* may allow macrophages to act as a sink for virus.

Infection of both classically and alternatively activated 129Sv/Ev macrophages resulted in up-regulation of TNF α , a cytokine associated with severe influenza virus infections *in vivo*. Macrophages can produce IFN γ (Gessani & Belardelli, 1998; Schroder *et al.*, 2004) and therefore it is likely that autocrine production of this cytokine contributes to the ability of alternatively activated macrophages to override the IL-4 response and produce an inflammatory response. Infection also resulted in upregulation of iNOS and IL12p40 in alternatively activated 129Sv/Ev macrophages. Type I interferons and IL-1 β can induce synthesis of iNOS (Gao *et al.*, 1998; Geller *et al.*, 1995) and it is likely these cytokines together with TNF α and IFN γ are involved in induction of iNOS and IL-12p40 following virus infection

(Drapier *et al.*, 1988; Farrell & Blake, 1996; Ma *et al.*, 1996). The upregulation of proinflammatory markers together with the down regulation of Arg-1 and CD206 expression demonstrates that infection results in a switch in cell phenotype. However, although both classes of macrophage produced a pro-inflammatory response following virus infection, alternatively activated macrophages produced lower levels of proinflammatory markers than classically activated macrophages. Thus, the data support the hypothesis that manipulation of the macrophage phenotype could have an impact on influenza virus pathogenesis.

IFN γ R^{-/-} macrophages were significantly compromised in pro-inflammatory responses and TNF α production was severely limited. Influenza virus infection did induce iNOS and IL-12p40 in these macrophages, most likely due to the action of type I interferons and IL-1 β , but levels were 1000 fold less than in wild type classically activated infected macrophages. Non-activated IFN γ R^{-/-} BMDMs have significantly higher expression of CD206 than 129Sv/Ev BMDMs. Given that CD206 has been shown to act as a receptor for influenza virus, it is interesting to speculate that this may play a role in the increased susceptibility to infection found in these macrophages. Overall, these data provide evidence IFN γ responsiveness is critical to the macrophage response to influenza virus infection and highlight the role of IFN γ as a critical cytokine in pathogenesis of influenza virus infections.

We have chosen to investigate the infection of BMDM and have successfully demonstrated that alternatively activated macrophages and those which cannot effectively mount a classical response (IFN γ R^{-/-}) are more susceptible to influenza virus infection. Whilst these studies were carried out with BMDMs rather than alveolar macrophages, they provide important clues for understanding the function of macrophages in control of influenza virus infections. Recently it has been shown that alternatively activated alveolar macrophages can protect against lethal challenge in a mouse model (Wang *et al.*, 2013). That study did not address macrophage

infection but our data would suggest that infection of alternatively activated macrophages per se is likely to play a role in this protective effect.

Macrophages clearly play a critical role in influenza virus infection. Depletion of macrophages in animal models leads to exacerbation of infection indicating their importance in an effective host response to infection (Tate *et al.*, 2011; Tumpey *et al.*, 2005). Similarly, transfer of macrophages accounts for the protective effect of prior infection with a herpesvirus (Saito *et al.*, 2013). However, they are major producers of inflammatory cytokines and hence have been implicated in the development of the severe pathology associated with fatal infections (Cheung *et al.*, 2002; Perrone *et al.*, 2008). Our study shows that macrophages of different phenotypes respond very differently to influenza virus infection. Given the diversity and plasticity of macrophages, understanding the interaction between macrophage phenotype and virus infection is likely to be fundamental to the development of novel strategies to prevent and treat severe influenza virus infections.

Methods

Cell culture

L929 murine fibroblasts were grown in tissue culture flasks (Nunc) in Rosewell Park Memorial Institute Medium (RPMI) supplemented with 10% foetal calf serum, 100units/ml penicillin, 100µg/ml streptomycin and 2mM L-glutamine (Invitrogen). Supernatant from confluent cultures was pooled, clarified by centrifugation at 8000g and stored in aliquots at -20°C as a source of M-CSF for bone marrow derived macrophages (BMDM).

Madin Darby canine kidney cells (MDCKs) were grown in Dulbecco's Modified Eagles Medium (DMEM, Invitrogen) supplemented as for RPMI.

Virus growth and assay

A/WSN/33 was propagated on MDCKs at a multiplicity of infection (MOI) of 0.001 for 48 hours before harvest of supernatant containing the virus. Supernatant was clarified by centrifugation at 3000g and stored in aliquots at -80°C. Titre was determined by plaque assay as previously described (Nicol *et al.*, 2012).

Macrophage isolation, activation and infection

129Sv/Ev and IFN γ R^{-/-} mice on the 129Sv/Ev background (Huang *et al.*, 1993) were purchased from B & K Universal and bred in-house. Femurs from 6-8 week old female mice were removed, cleaned in alcohol and the bone marrow flushed out with supplemented RPMI, using a 25G needle and syringe. Bone marrow cells were plated onto 100mm bacteriological dishes (Sterilin) in 50% supplemented RPMI: 50% L929 fibroblast conditioned media (complete macrophage media). Cultures were initially set up by seeding cells from one femur in each plate. Bone marrow derived macrophages were passaged on day four by the following method: medium was removed and retained, the adherent cells were incubated with Dulbecco's Phosphate Buffered Saline (D-PBS; Life Technologies) for 5 minutes then detached from the plastic by washing vigorously with D-PBS using an 18 gauge needle and

syringe and recovered by centrifugation at 8000g. Cells from each plate were then reseeded into two new plates in complete macrophage medium supplemented with 5ml original medium. On day 7, BMDM were harvested, counted and seeded into 100mm dishes, 96 well plates or onto 8 well glass chamber slides (BD Falcon) at 1×10^5 cells/ml. BMDM were activated with either 1ng/ml IFN γ or 4ng/ml IL-4 (Peprotech) in complete macrophage media for 16 hours prior to infection with A/WSN/33. Activating media was removed from activated BMDM and kept at 37°C while infection was carried out. BMDM were infected at an MOI of 10 by incubation with virus diluted in serum free DMEM for 1 hour. The inoculum was then removed and activating media replaced onto the cells to allow continued exposure to cytokine. After 48 hours, medium was removed for assay of iNOS and Arg-1 the cells were washed by incubation in Ca $^{2+}$ Mg $^{+}$ free PBS (Invitrogen) for 5 minutes, flushed from the dishes as described above, counted and pelleted by centrifugation at 1500g. Cell pellets were stored at -80°C.

Cell viability assays

Activated and non-activated BMDMs in 96 well plates were infected at an MOI of 10 and, at appropriate time points, cell viability was measured using the CellTiter-Blue cell Viability Assay (Promega). Samples were assayed in triplicate.

Immunostaining

Chamber slides were washed with PBS and fixed for 30mins with 4% (w/v) paraformaldehyde (PFA). After fixation, slides were washed with PBS and either stained immediately or stored at 4°C until required. Before staining, stored slides were washed with PBS and blocked for 30mins at room temperature with CAS block (Invitrogen). After extensive washing, slides were probed with 1 in 500 dilution of polyclonal goat anti-influenza A H1N1 strain USSR antibody (AbD Serotec) in CAS block. After 30 minutes incubation at room temperature, slides were washed with PBS and bound antibody was detected by incubation for 30 minutes with rabbit anti-goat/sheep alexafluor-488 conjugated secondary antibody, diluted 1 in 1000 in CAS block

(Invitrogen). Unbound conjugate was removed by washing with PBS and the slides were counterstained with DAPI and mounted in Prolong Gold (Life Technologies) mounting medium.

iNOS assays

Active inducible nitric oxide synthase (iNOS) was determined by the Greiss reagent bioassay, which results in production of nitrite and a colour change from colourless to pink in the presence of enzyme. 100µL Greiss reagent, 5.8% (v/v) H₃PO₄, 1% (w/v) sulphanilamide, 0.1% (w/v) N-(1-Naphthyl) ethylenediamine dihydrochloride was added to 100µL BMDM supernatant or 100µL sodium nitrite standard (Sigma) and absorbance read at 540nm.

Arginase-1 bioassay

Bioactive Arginase-1 was measured by conversion of L-Arginine to urea as follows. 1x10⁵ BMDM were plated onto 96 well flat bottomed plates (Nunc), washed with PBS and lysed with 0.1% Triton-X (Sigma). The lysate was then removed to sterile 1.5ml tubes. After addition of 100µL 25mM Tris-HCl and 20µL 10mM MnCl₂, tubes were incubated at 56°C for 10 minutes. 100µL of each sample was transferred to fresh tubes and incubated with 100µL 0.5M L-Arginine for 2 hours. During this time a standard dilution series of urea was made. Following the incubation step, 800µL 10% (v/v) sulphuric/30% (v/v) phosphoric acid solution was added along with 40µL isonitropropionophenone, mixed by vortexing and incubated at 95°C for 30 minutes. Once cooled, samples and standards were placed in a 96 well plate and absorbance read at 540nm.

Quantitative RT- PCR

RNA was extracted from frozen BMDM using an RNeasy minikit and Qiashredders (Qiagen), as per manufacturer's guidelines. Genomic DNA was removed by treatment with DNA-free (Ambion) according to the manufacturer's instructions. 1-2µg RNA was reverse transcribed to cDNA with Superscript III (Invitrogen). cDNA was routinely diluted 1 in 20 for quantitative reverse transcriptase PCR (qRT-PCR) analysis. Primers were designed as follows for each

gene of interest, along with reference genes succinate dehydrogenase A (SDHA) and calnexin (CNX). SDHA and CNX were chosen from a panel of 12 housekeeping genes (Quantace) which were tested to determine the genes with the most stable expression in BMDMs. Optimal amplification conditions were determined for each gene of interest to ensure >95% efficiency of single products. qRT-PCR was carried out using a Rotorgene 3000 cyclor (Qiagen). Primers sets are as follows: calnexin F ttagttgaccagtctgttg, R cctttcatcccaatcttcag; succinate dehydrogenase A F gctcctactgatgaaacctg, R aactcaatcccttacagcaa; iNOS F tgctactgagacagggaag, R gacagtctccattcccaa; TNF α F caccaccatcaaggactcaa, R gagagaggcaacctgaccac; IL-12p40 F ggaagcacggcagcagaata, R ttgagggagaagtaggaatgg; M1 F ctctctatcgtcccgtcagg, R gagcgtgaacac aaatccta.

Each gene of interest was normalized to the reference genes using Genex software (MultiD) and relative expression of infected to mock controls was calculated.

Statistical methods

Statistical analysis of the differences in the various parameters of interest (the percentage of macrophages infected, normalised copy number, percentage survival and gene expression) following classical and alternative activation of macrophages in both 129Sv/Ev and IFN γ R^{-/-} mice was performed using standard linear-mixed effect models (Pinheiro & Bates 2009), with individual mouse entered as the random effect to take account of the repeated measures taken from them. All statistical analyses were carried out on log₁₀ transformed data to normalise the residuals and were performed in R (v 3.1.1 © 2014 The R Foundation for Statistical Computing), using the package 'nlme' (v 3.1-117). Statistical significance was graded as * P<0.05, ** P<0.005, *** P<0.001.

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Figure legends

Figure 1. Influenza virus infection of 129Sv/Ev BMDMs. BMDM cultured from femurs of female 129Sv/Ev mice were mock-infected or infected with A/WSN/33 and stained with antiserum to viral antigens (a) mock-infected cells; (b) 1h post-infection, MOI 10; (c) 6h post-infection, MOI 10; (d) 48h post-infection, MOI 10.

Figure 2. Treatment of 129Sv/Ev bone marrow derived macrophages with IFN γ or IL-4 leads to polarization of the macrophages to classical or alternative phenotypes. BMDMs derived from 129Sv/Ev and IFN γ R^{-/-} mice were treated for 16h with 1ng/ml IFN γ or 4ng/ml IL-4. Expression of Arg-1 (a) or iNOS (c) mRNA was measured by qRT-PCR or by biochemical assay for Arg-1 activity (b) or iNOS activity (d).

Figure 3. Alternatively activated macrophages are more readily infected with A/WSN/33 than classically activated macrophages. BMDMs derived from 129Sv/Ev (a-e) and IFN γ R^{-/-} (f-j) mice were cultured in medium containing M-CSF alone (a,f) or treated with IFN γ (b,g) or IL-4 (c,h) for 16h and infected with 10pfu/cell A/WSN/33 followed by staining with antiserum to virus antigens. The percentage of cells positive for antigen was quantitated at 6h (d,i) and 48h (e,j) * p<0.05 *** p<0.001.

Figure 4. Alternatively activated BMDMs produce more M1 mRNA than classically activated macrophages. BMDMs were activated with IFN γ (classically activated) or IL-4 (alternatively active) and infected with 10pfu/cell A/WSN/33. 48h post-infection, M1 mRNA levels were measured by qRT-PCR. *** indicates p<0.001.

Figure 5. Alternatively activated BMDMs are more susceptible to cell death following infection with A/WSN/33 but do not support higher levels of virus replication. BMDMs from 129Sv/Ev and IFN γ R^{-/-} mice were untreated or activated with IFN γ or IL-4 and infected with 10pfu/cell. Cell viability was measured by the CellTiter-Blue assay at various times after infection. (a) 129Sv/Ev BMDMs; (b) IFN γ R^{-/-} BMDMs, *** p<0.001. Virus titres in cell

supernatants at 1h and 48h post-infection were measured by titration on MDCK cells (c).
Results are representative of 3 independent experiments.

Figure 6. Expression of pro- and anti-inflammatory markers in classically and alternatively activated macrophages infected with influenza virus A/WSN/33. BMDMs were treated with 1ng/ml IFN γ or 4ng/ml IL-4 for 16hours and then infected with 10pfu/cell A/WSN/33. Cells were harvested at 48 hours post-infection and expression of cellular markers was monitored by qRT-PCR. Results are representative of 3 independent experiments. (a,b) INOS; (c,d) Arg-1; (e,f) TNF α ; (g,h) CD206. * p<0.05; ** p<0.005, ***p<0.001.

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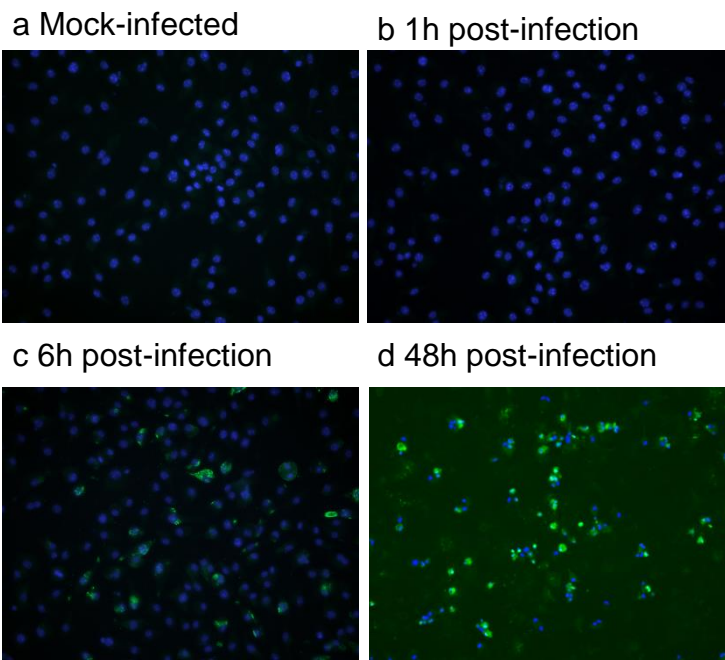
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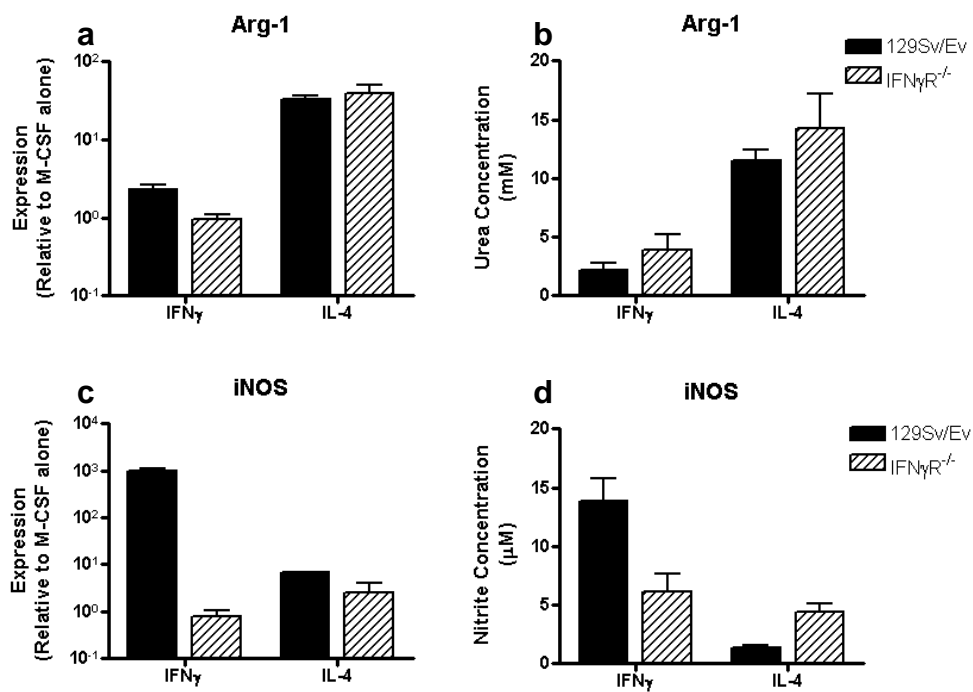
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Figure 1

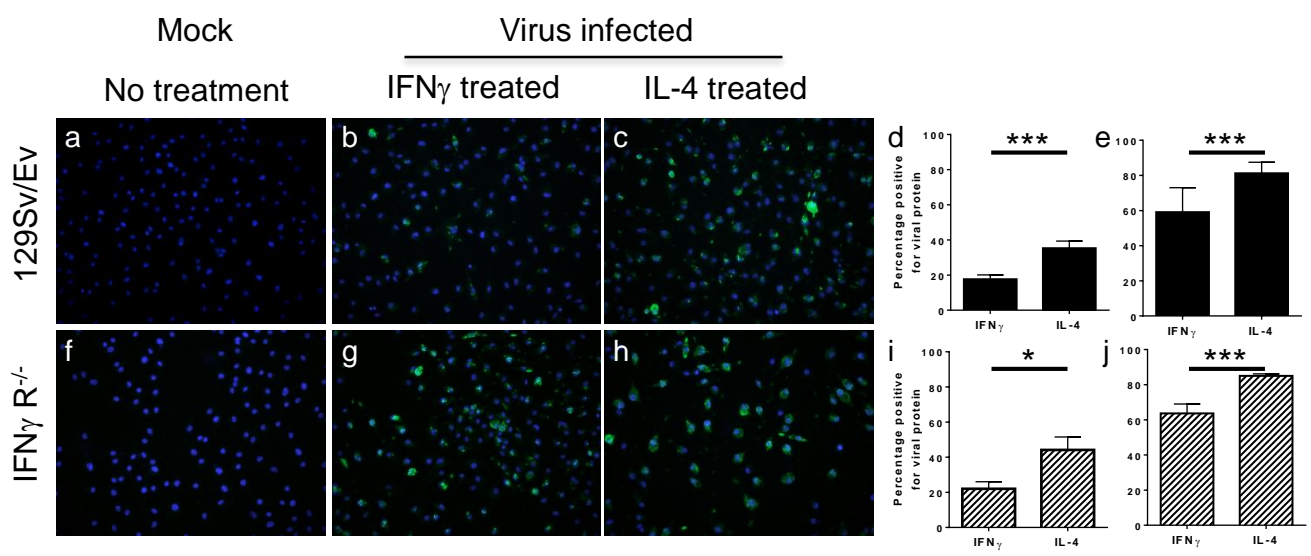


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Figure 2

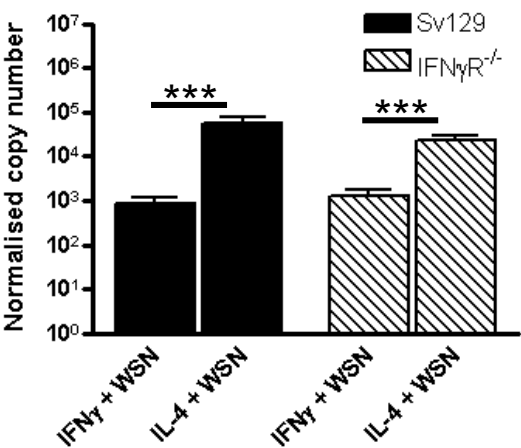


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Figure 3



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Figure 4



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Figure 5

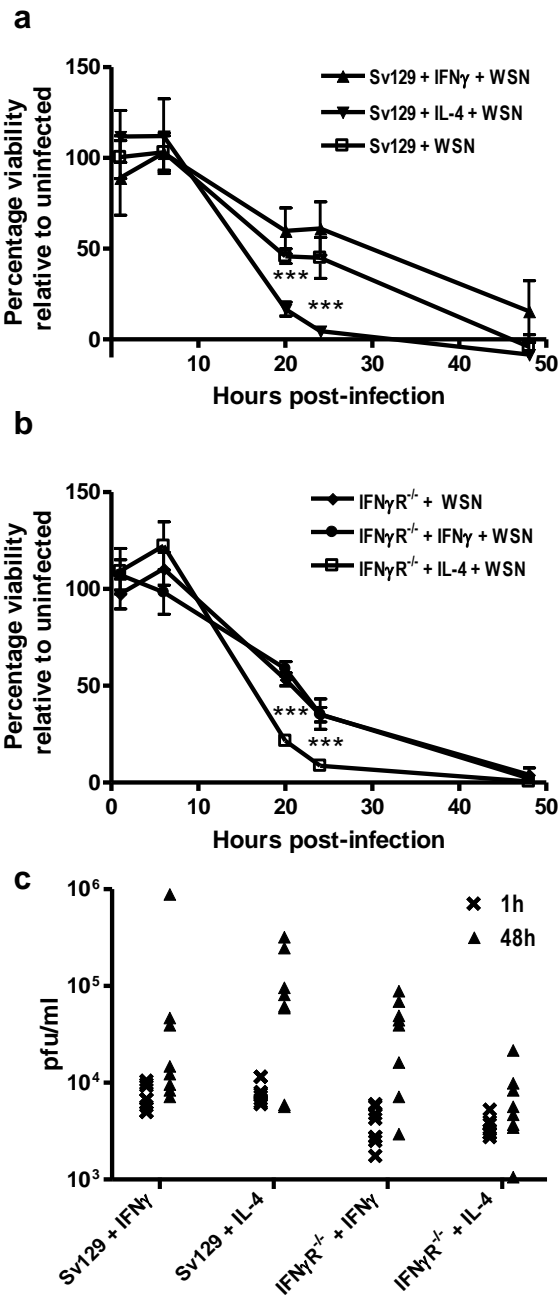


Figure 6

